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Original Article

HPLC-DAD-MS/MS profiling of phenolics from *Securigera securidaca* flowers and its anti-hyperglycemic and anti-hyperlipidemic activities

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ABSTRACT

Securigera securidaca (L.) Degen & Döefl., Fabaceae, has been widely used in the Iranian, Indian and Egyptian folk medicine as antidiabetic and anti-hyperlipidemic remedy. Phenolic profiling of the ethanolic extract (90%) of the flowers of *S. securidaca* was performed *via* HPLC-DAD-MS/MS analysis in the positive and negative ion modes. The total polyphenols and flavonoids in the flowers were determined colorimetrically, and the quantification of their components was carried out using HPLC-UV. Total phenolics and flavonoids estimated as gallic acid and rutin equivalents were 82.39 ± 2.79 mg/g and 48.82 ± 1.95 mg/g of the dried powdered flowers, respectively. HPLC-DAD-MS/MS analysis of the extract allowed the identification of 39 flavonoids and eight phenolic acids. Quantitative analysis of some flavonoids and phenolics (mg/100 g powdered flowers) revealed the presence of isoquercetrin (3340 ± 2.1), hesperidin (32.09 ± 2.28), naringin (197.3 ± 30.16), luteolin (10.247 ± 0.594), chlorogenic acid (84.22 ± 2.08), catechin (3.94 ± 0.57) and protocatechuic acid (34.4 ± 0.15), in the extract. Moreover, the acute toxicity, hypoglycemic and hypolipidemic effects of the extract were investigated using alloxan induced diabetes in rats in a dose of 100, 200, and 400 mg/kg bwt. The ethanolic extract was safe up to a dose of 2000 mg/kg. All tested doses of the flower extract showed marked decrease in blood glucose level by 31.78%, 66.41% and 63.8% at 100, 200 and 400 mg/kg bwt, respectively, at $p < 0.05$. Regarding the anti-hyperlipidemic effect, a dose of 400 mg/kg of the flower extract showed the highest reduction in serum triacylglycerides and total cholesterol levels (68.46% and 51.50%, respectively at $p < 0.05$). The current study proved the folk use of the flowers of *S. securidaca* as anti-diabetic and anti-hyperlipidemic agent which could be attributed to its high phenolic content.

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Introduction

Diabetes mellitus is a complex disorder that characterized by chronic hyperglycemia and disturbances of fat and protein metabolism associated with malfunction in insulin secretion and/or insulin action. The utilization of impaired carbohydrate leads to accelerated lipolysis, resulted in hyperlipidemia (Kim et al., 2006). The Middle East and Northern Africa has the highest prevalence of diabetes as a world region with 34 million diabetic persons according to international diabetes federation (IDF Diabetes Atlas, 2011).

In recent years, there is growing evidence that plant polyphenols including flavonoids are unique nutraceuticals and supplementary treatments for various aspects of type 2 diabetes mellitus. Plant polyphenols can modulate carbohydrate and lipid metabolism, attenuate hyperglycemia, dyslipidemia, insulin resistance, alleviate oxidative stress and prevent the development of long-term diabetic complications (Bahadoran et al., 2013). Nowadays, there is a growing interest in the analysis and identification of medicinal plants' phenolic constituents aiming at finding new sources for these compounds and to establish their structure activity relationship.

Securigera securidaca (L.) Degen & Dörfl., Fabaceae, has been widely used in the Iranian, Indian and Egyptian folk medicine as antidiabetic and anti-hyperlipidemic remedy (Ali et al., 1998; Azarmiy et al., 2009; Porchezhian and Ansari, 2001). Chloroformic

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extract of *S. securidaca* decreased fasting serum glucose level, increased food consumption, body weight and glycogen content of the liver in rats (Zahedi-Asl et al., 2005). Total aqueous extract of the seeds showed significant decrease in blood glucose level (−35%) in glucose loaded mice (Ali et al., 1998). In addition, *S. securidaca* seed suspension has a protective effect against alloxan-induced hyperglycemia and oxidative stress in rats (Mahdi et al., 2011). Hydroalcoholic extract of *S. securidaca* seeds produced a significant reduction in the level of triglyceride, LDL as well as decrease in lipid peroxidation (Fathi et al., 2010). Total seed extract improved endothelium dependent vasodilation in high fat fed rats by lowering lipid formation around the aorta in hypercholesterolemic rats and decreasing atherosclerotic lesions (Azarmiy et al., 2009). The hypoglycemic effect of the seed was estimated to be related to its flavonoid content (Hosseinzadeh et al., 2002). Concerning the flowers of *S. securidaca*, two flavonoids, kaempferol and astragalin were isolated from its aqueous extract (Ali et al., 1998) but no reports were found regarding the effect of the flowers on plasma glucose and lipids in diabetic rats.

In the present study, the acute toxicity, anti-diabetic, anti-hyperlipidemic effect of the alcoholic extract of *S. securidaca* flowers were evaluated in alloxan induced diabetes model in rats. The total polyphenols and flavonoids in the flowers were determined and the phenolic composition of the extract was described using HPLC/DAD (high-performance liquid chromatographic/diode array detector) coupled with ESI-MS (electrospray ionization/mass spectrometry) to identify the major phenolic compounds present in the extract. In addition, HPLC-DAD quantification of major phenolic acids and flavonoids was carried out.

Material and methods

Chemicals

Reagents for spectrophotometric determination of phenolic compounds, Folin–Ciocalteu's reagent: was obtained from Loba-Chemie (Mumbai, India), sodium carbonate and *t*-butyl hydroquinone were obtained from Sigma, USA.

Regarding HPLC analysis of phenolic compounds; acetonitrile and methanol used were of HPLC grade, and were purchased from Sigma–Aldrich (Steinheim, Germany). *o*-Phosphoric acid used was of analytical grade from Sdfine Chemlimited (Mumbai, India) and formic acid was purchased from E-Merck (Darmstadt, Germany). Distilled water was further purified using a Milli-Q system (Millipore, MA, USA). Acetonitrile and acidulated water were filtered through a 0.45 pm membrane filter (Pall Gelman Laboratory, USA) and degassed in an ultrasonic bath prior to HPLC analysis. Isoquercetrin, rutin, gallic acid, *trans*-cinnamic acid, salicylic acid, naringin, protocatechuic acid, ellagic acid, luteolin, quercetin, caffeic acid, hesperetin, *p*-coumaric acid, kaempferol and hesperidin were purchased from sigma Co. (St. Louis, MO, USA).

Plant material

Samples of *Securigera securidaca* (L.) Degen & Dörfl, Fabaceae, were collected during the years (2010–2013) from The Experimental Station of Medicinal and Aromatic Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza. Plant was kindly authenticated by Botany specialist, Dr. Mohamed El-Gebaly, Department of Botany, National Research Center (NRC), Giza, Egypt and a voucher specimen was kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University (no. 11-6-2013-2).

Determination of total phenolics and flavonoids contents

Quantification of phenolic compounds was carried out using Folin–Ciocalteau's method as reported by Siger et al. (2008). Briefly; 1 g of dried powdered flowers was homogenized with 40 ml of 80% methanol using a pestle and mortar, filtered through Watmann No. 1 filter paper and transferred into a volumetric flask (100 ml) with 80% methanol. 0.2 ml of the methanolic extract was placed in a volumetric flask (10 ml) and 0.5 ml Folin–Ciocalteu reagent (2 N) was added. After 3 min, saturated sodium carbonate (1 ml) (20% in distilled water) was added and the volume was completed with distilled water. After 1 h, absorbance of blue color was measured at λ_{max} 725 nm against a blank (distilled water) using Unicam UV–visible Spectrometer. Gallic acid was used to compute the standard calibration curve (20, 40, 60, 80, 100 mg/ml). Determinations were carried out in triplicates; results were represented as the mean values ± standard deviations and expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g D.W.).

Total flavonoids were extracted according to the method of Hertog et al. (1992), 1 g of dried powdered flowers was homogenized with 40 ml of 62.5% methanol with 0.1 g *t*-butyl hydroquinone (w/v) and 10 ml of 6 N hydrochloric acid was added carefully and the mixture refluxed at 90 °C for 2 h. After cooling, the supernatant was filtered and transferred to a volumetric flask (100 ml) with 62.5% methanol. Sample (1 ml), Folin–Ciocalteau's reagent (2 N) (0.5 ml) and Na_2CO_3 (200 mg/ml) (3 ml) were added, vortexed and then allowed to stand for 15 min at room temperature in a dark place and absorbance was measured at 725 nm. Rutin was used as standard and the equivalents (w/w) were determined from a standard concentration curve (20, 40, 60, 80, 100 mg/ml) (Meenakshi et al., 2009). Determinations were carried out in triplicates; results were represented as the mean values ± standard deviations and expressed as mg rutin equivalents (RE) per gram dry weight.

Extraction procedures

The air-dried powdered *S. securidaca* flowers (1.6 kg) were exhaustively extracted with 90% ethanol by cold maceration. The total alcoholic extract was combined and evaporated under reduced pressure at a temperature not exceeding 50 °C, yielding 250 g of dry residue. For biological study the extract dissolved in bi-distilled water by the aid of an ultrasonic bath just prior to the investigation.

Preparation of the extract for HPLC-DAD-MS/MS analysis

The previously prepared ethanolic extract of the flowers (20 mg) was dissolved in HPLC grade methanol (2 ml). The methanolic extract was placed in ultrasonic bath for 5 min and filtered through 0.4 μm membrane filter. Aliquot of 10 μl was injected into the LC/DAD/MS analysis system.

HPLC-DAD-ESI–MS apparatus

The analysis was performed using a Hewlett-Packard 1100 (Waldbronn, Germany) composed of a quaternary pump with an on line degasser, a thermostated column compartment, a photodiode array detector (DAD), an auto sampler, and 1100 ChemStation software, coupled with electrospray ionization (ESI) interfaced Bruker Daltonik Esquire-LC ion trap mass spectrometer (Bremen, Germany) and an Agilent HP1100 HPLC system equipped with an autosampler and a UV–vis absorbance detector.

Conditions for HPLC-DAD-MS/MS analysis of flavonoids

The HPLC separation was performed on Eclipse XDB C18 column (50 mm × 2.1 mm, 1.8 μm, Agilent Company, USA). Mobile phase consisted of two solvents, (A) methanol and (B) 0.2% formic acid. Separation of compounds was carried out with gradient elution profile: 0 min, A: B 10:90; 36 min, A: B 70:30; 50 min, A: B 100:0; 60 min. Chromatography was performed at 30 °C with a flow-rate of 0.8 ml/min. UV traces were measured at 254, 360 and UV spectra (DAD) were recorded between 190 and 900 nm.

Mass spectrophotometric conditions

The ionization parameters were as follows: capillary voltage 4000 V, end plate voltage −500 V; nebulizing gas of nitrogen at 35.0 p.s.i.; drying gas of 10 l/min nitrogen at 350 °C. Mass analyzer scanned from 50 to 1300 μ. The MS–MS spectra were recorded in auto-MS–MS mode. The fragmentation amplitude was set to 1.0 V. Mass spectra were simultaneously acquired using electrospray ionization in the positive and negative ionization modes.

Quantitative determination of phenolic compounds by HPLC

Quantitative determination of phenolic compounds was performed using HPLC apparatus, Agilent Series 1200 apparatus (Agilent, USA) equipped with autosampling injector, solvent degasser, quaternary HP pump (series 1200) and ultraviolet (UV) detector (set at 280 nm for phenolic acids and 330 nm for flavonoids). The analysis was achieved on a zobrax ODS C18 column (particle size 5 μm, 250 mm × 4.6 mm Ø). Column temperature was maintained at 35 °C. Flavonoid separation was done adopting the method of Mattila et al. (2000), using a mobile phase consisting of 50 mM H_3PO_4, pH 2.5 (solution A) and acetonitrile (solution B) acetic acid (40:60, v/v) in the following gradient: isocratic elution 95%A:5%B, 0–5 min; linear gradient from 95%A:5%B to 50%A:50%B, 5–55 min; isocratic elution 50%A:50%B, 55–65 min; linear gradient from 50%A:50%B to 95% A:5%B, 65–67 min. The flow rate of the mobile phase was 0.7 ml/min. Phenolic acids separation was done adopting the method of Goupy et al. (1999) with a solvent system consisting of A (aqueous acetic acid 2.5%), B (aqueous acetic acid 8%) and C (acetonitrile) in the following gradient: at 0 min, 5% B; at 20 min, 10% B; at 50 min, 30%B; at 55 min, 50%B at 60 min, 100%B; at 100 min, 50% B and 50% C; at 110 min, 100% C until 120 min. The solvent flow rate was 1 ml/min. The injection volumes were 5 μl. Standard flavonoids and phenolic acids were prepared as 10 mg/50 ml solutions in methanol and they were diluted to make concentrations (20–40 μg/ml) and injected into HPLC. Quantification of compounds was performed based on peak area computation (external standard method). The analysis was run in triplicates and the concentrations of the identified compounds were expressed as (mg ± SD/100 g dry weight) and listed in Table 2.

Experimental animals

Adult Wister male albino rats, weighing 180–250 g, were obtained from the Animal House Colony of the National Research Center (Dokki, Giza, Egypt), and were housed under conventional laboratory conditions throughout the period of experimentation. The animals were fed a standard rat pellet diet and allowed free access to water.

Drugs and kits

Alloxan monohydrate powder (Sigma–Aldrich, St. Louis, MO, USA), Gliclazide (Servier, Egypt) were used in the present investigation. The biochemical kits used in the study were glucose kits (Biodiagnostic, Egypt), cholesterol kits (Biodiagnostic, Egypt) and triacylglycerides kits (Biodiagnostic, Egypt).

Acute toxicity

Acute oral toxicity of the ethanolic extract of the flowers of *S. securidaca* L. was performed following the method of Lorke (1983).

Anti-hyperglycemic activity and anti-hyperlipidemic activity

Rats were weighed and injected intraperitoneally with alloxan (150 mg/kg) dissolved in distilled water. After 48 h blood samples were withdrawn from the retro-orbital venous plexus under light ether anesthesia and the serum was separated by centrifugation for the determination of glucose level. Only rats with serum glucose levels more than 250 mg/dl were selected and considered as hyperglycemic animals according to method of Neshwari et al. (2012). The hyperglycemic rats were then divided into five groups (10 rats each). The first group of diabetic rats served as control; the second to fourth groups received alcoholic extract of the flowers at doses 100, 200 and 400 mg/kg orally for 10 consecutive days; and the fifth group of diabetic rats received Gliclazide (antidiabetic standard) at dose of 5 mg/kg bwt orally for 10 consecutive days. The extract and Gliclazide were started 48 h after alloxan injection at which time hyperglycemia was confirmed. Twenty-four hours after the last dose of either drug treatment, a blood sample was withdrawn from the retro-orbital venous plexus from 18 h food-deprived rats and was centrifuged at 3000 rpm for 10 min. The serum was obtained for determination of the serum glucose level, triacylglycerides and total cholesterol level.

Determination of serum glucose level

Glucose level was determined as quinineamine using a test reagent kit (Biodiagnostic, Egypt) according to the method of Trinder (1969). The absorbance was measured at 510 nm and the results were expressed as mg/dl.

Determination of serum triacylglyceride level

Triacylglycerides were estimated by enzymatic methods using diagnostic kit (Biodiagnostic, Egypt) according to the method of Fossati and Prencipe (1982). The absorbance was measured at 510 nm and the results were expressed as mg/dl.

Determination of serum total cholesterol level

Total cholesterol was estimated by enzymatic methods using diagnostic kit (Biodiagnostic, Egypt) according to the method of Allain et al. (1974). The absorbance was measured at 500 nm and the results were expressed as mg/dl.

Statistical analysis

Statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Tukey test. Results are expressed as means ± SEM (n = 10).

Results and discussion

Total phenols and flavonoids contents of *S. securidaca* flowers were investigated. The value of total phenolics was 82.39 ± 2.79 mg gallic acid equivalent (GAE)/g (D.W.) and that of the total flavonoids was 48.82 ± 1.95 mg rutin equivalent (RE)/g (D.W.).

Table 1
Peak assignmenet of metabolites in ethanolic extract of *S. securidaca* using LC-DAD/MS in positive and negative ionization modes.

No.	RT	[M−H]$^-$ *m/z*	[M+H]$^+$ *m/z*	Fragment ions	Identified compounds
1	0.9	147	–	147, 62	*trans*-Cinnamic acid
2	1.2	–	139	137, 93	Salicylic acid
3	1.5	–	216	170 [(M+H)-HCOOH], 125, 97	Gallic acid
4	1.8	–	152	153, 109	Protocatechuic acid
5	2.8	–	292	289, 271, 245, 227, 205, 179, 125	Catechin
6	11.1	447	–	327[(M−H)-120], 285[(M−H)-162], 284, 255, 227	Kaempferol-7-O-glucoside
7	11.7	448	–	447, 357 [(M−H)-90], 327[(M−H)-120], 285[(M−H)-162]	Orientin
8	12.4	625	–	505[(M−H)-120], 463[(M−H)-162],300, 301[(M−H)-162]	Quercetin-3,7-di-O-glucoside
9	12.7	447	–	327[(M−H)-120], 284[(M−H)-H-162],255 [aglycone-2H-CO], 243, 241, 217, 213, 199, 175, 149	Luteolin-3′-O-glucoside
10	12.8	447	–	327[(M−H)-120], 285[(M−H)-162], 284, 257[aglycone-CO], 243, 241, 217, 213, 199, 175, 149	Luteolin-7-O-glucoside
11	13.2	593	–	503[(M−H)-90], 473[(M−H)-120], 431[(M−H)-162], 311[(M−H)-120–162], 283	Isovitexin-4′-O-glucoside
12	13.6	609	–	489[(M−H)-120], 447[(M−H)-162], 357[(M−H)-90–162], 327[(M−H)-120–162]	Isoorientin-4′-O-glucoside
13	14.2	593	–	575 [(M−H)-18], 503[(M−H)-90], 473[(M−H)-120], 383[(M−H)-90–120], 353[(M−H)-120–120]	Apigenin 6,8-di-C-glucoside (vicenin-2)
14	14.9	593	–	503[(M−H)-90], 473[(M−H)-120], 431, 341, 311 [(M−H)-120–162]	Isovitexin-7-O-glucoside (saponarin)
15	15	–	595	449, 433, 284	Kaempferol-O-neohesperidosideee
16	15.4	623	–	503[(M−H)-120], 461[(M−H)-162], 341[(M−H)-120–162], 315[(M−H)-146–162], 297, 195, 179, 161, 153, 135	Isorhamnetin-3-O-glucoside-7-O-rhamnoside
17	15.9	448	–	447, 429, 357 [(M−H)-90], 327 [(M−H)-120], 285[(M−H)-162]	Iso-orientin
18	15.9	564	–	545, 503[(M−H)-60], 473[(M−H)-90], 443[(M−H)-120], 425, 413, 383, 353[(M−H)-120–90],	Apigenin-6-C-pentoside–8-C-hexoside
19	16.1	639	–	639(M−H)-, 519[(M−H)-120], 477[(M−H)-162], 459 [(M−H)-180], 357[(M−H)-120–162], 314, 315[(M−H)-162–162]	Isorhamnetin-O-sophoroside
20	16.3	593	–	503 [(M−H)-90], 473 [(M−H)-120], 447 [(M−H)-146], 429 [(M−H)-146-H_2O], 413, 395, 383, 353, 329, 299	Isoorientin-2″-O-rhamnoside
21	17.3	431	–	431, 353, 341[(M−H)-90], 311[(M−H)-120], 269	Isovitexin
22	17.6	577	–	577, 413[(M−H)-146–18], 457[(M−H)-120], 341, 311, 293[aglycone + 41–18]-, 173	Isovitexin-2″-O-rhamnoside
23	17.7	–	580	579, 459, 271, 235	Naringin
24	17.9	463	–	301[(M−H)-162], 300, 271, 255, 179, 151	Isoquercetrin
25	18.1	–	303	301, 258, 143	Hesperetin
26	18.1	–	464	343, 301, 179, 151	Hyperoside
27	18.2	–	303	257, 229, 185	Ellagic acid
28	18.9	578	–	431[(M−H)-146], 308, 285, 269[(M−H)-162–146]	Apigenin-7-O-rutinoside
29	19.2	608	–	301, 281, 237, 326	Hesperidin
30	19.3	577	–	431[(M−H)-146], 285[(M−H)-146–146]	Kaempferol-3,7-dirhamnoside (kaempferitrin)
31	19.5	–	609	301, 464, 179, 151	Rutin
32	19.5	448	–	285[(M−H)-162], 284, 255, 267, 257, 256, 241, 229, 213, 163	Kaempferol-3-O-glucoside (astragalin)
33	19.5	607	–	608[(M−H)-H], 463[(M−H)-146], 447[(M−H)-162], 299[(M−H)-162–146]	Quercetin-3-O-glucoside-7-O-rhamnoside
34	19.7	–	287	287, 285, 217, 241, 175	Luteolin
35	19.7	593	–	593 (M−H), 447[(M−H)-146], 285[(M−H)-146–162]	Kaempferol-3-O-glucoside-7-rhamnoside
36	19.9	477	–	357[(M−H)-120], 315[(M−H)-162], 314, 286, 285, 271, 243, 299	Isorhamnetin-3-O-glucoside
37	20.1	479	–	357[(M−H)-120], 315[(M−H)-162], 314, 286, 285, 271, 299	Isorhamnetin-7-O-glucoside
38	23.3	479	–	477, 301[(M−H)-176], 273, 257, 179, 193, 151	Quercetin-3-glucuronide
39	24.9	625	–	449, 461[(M−H)-162], 447[(M−H)-176], 337, 287, 285[(M−H)-162–176]	Luteolin-7-O-glucuronide-3-O-glucoside
40	25.4	609	–	449[(M−H)-162], 431[(M−H)-180], 301, 287[(M−H)-162–162]	Luteolin di-O-glucoside
41	26.4	563	–	440 [(M−H)-120], 323, 269[(M−H)-132–162]	Apigenin-O-pentosyl-hexoside
42	33	–	301	301, 151, 179	Quercetin
43	34.3	–	286	285, 257, 151, 169, 241	Kaempferol
44	38.5	–	179	179, 135, 107	Caffeic acid
45	38.5	593	–	473[(M−H)-120], 447[(M−H)-146], 301[(M−H)-146–146], 299	Quercetin-3,7-dirhamnoside
46	38.8	–	353	353, 191, 190	Chlorogenic acid
47	43.5	–	165	163, 119	*p*-Coumaric acid

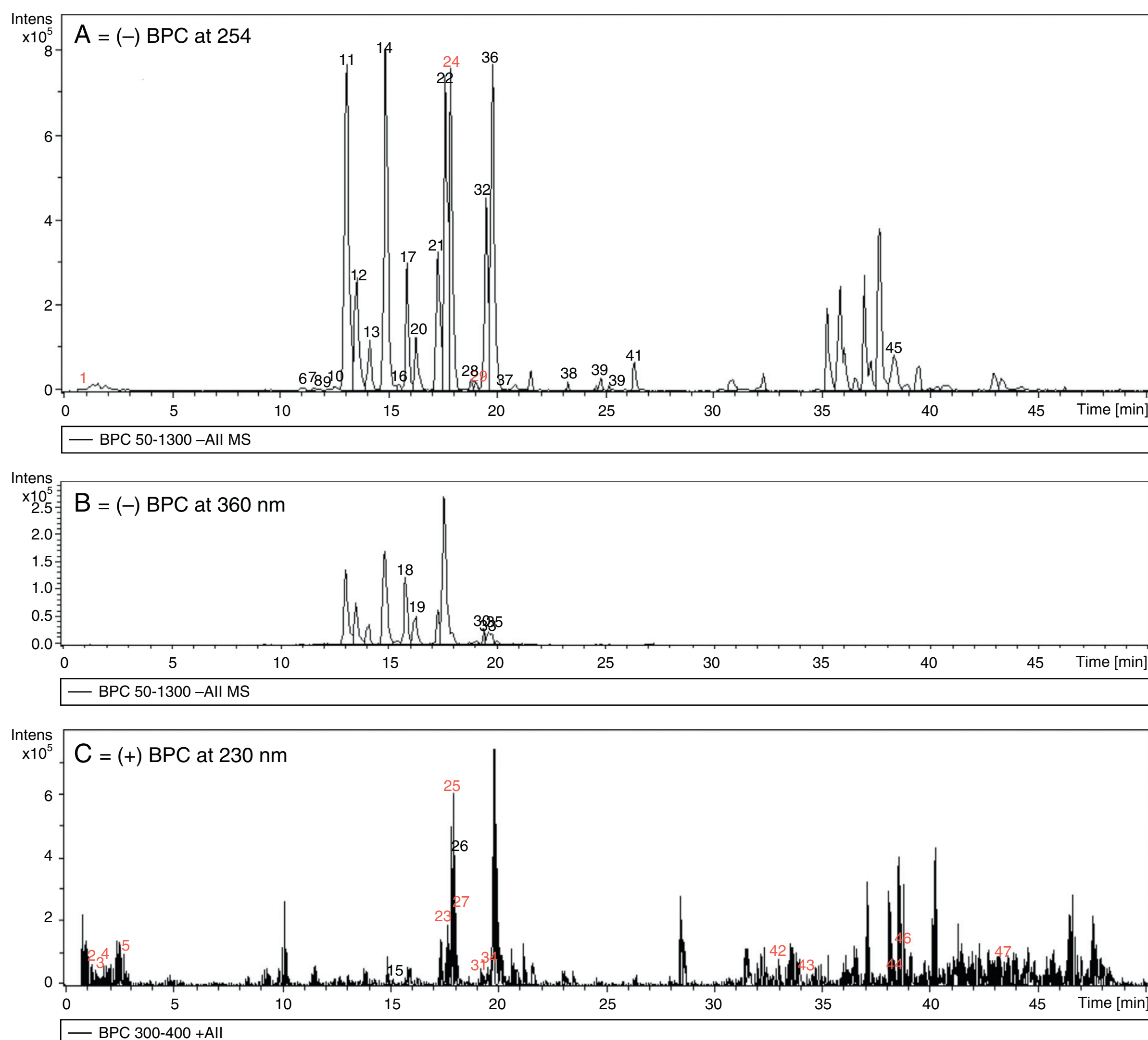

Fig. 1. HPLC-ESI-MS/MS base peak chromatograms (BPC) of the crude ethanolic extract of the flowers of *S. securidaca* L. recorded at 254 nm (A) and 360 nm (B), negative ion mode, and at 230 nm (C), positive ion mode. Peak numbers follow those listed in Table 1.

HPLC-DAD-MS/MS analysis of phenolic compounds

The HPLC-DAD-MS/MS analysis was carried out in both negative and positive ionization modes. The HPLC-DAD base peak chromatograms (BPC) recorded at 254 nm (A), 360 nm (B) in the negative mode and BPC in the positive mode obtained at 230 nm (C) are shown in Fig. 1. The identities, retention times and observed molecular and fragment ions for individual compounds are presented in Table 1.

A total of 47 phenolic compounds have been tentatively identified by comparing retention times and MS data of the detected peaks with that reported in the literature and by searching phytochemical dictionary of natural products database (CRC). Identified compounds belonged to various classes (Table 1) including eight phenolic acids and 39 flavonoids. The phenolic acids included three hydroxybenzoic acids (salicylic acid, gallic acid and protocatechuic acid) and four hydroxycinnamic acid derivatives (*trans*-cinnamic acid, caffeic acid, chlorogenic acid and *p*-coumaric acid), in addition to ellagic acid. Flavonoids were present mostly as flavones (including both *O*- and *C*-glycosides with apigenin or luteolin as aglycone) and flavonols (derived from the aglycones; quercetin, kaempferol and isorhamnetin), flavanones (naringenin and hesperitin and their glycosides). While, only one flavan-3-ol (catechin) was identified. Sugar moieties consists of hexosides, deoxyhexosides and pentosides as deduced from the loss of 162 μ, 146 μ and 132 μ, respectively.

Identification of flavonoids

Seventeen flavones were identified on the basis of their MS/MS fragmentation. Three compounds were mono-*C*-glycosyl flavones (peaks 7, 17 and 21) producing MS fragmentation patterns characteristic to *C*-glycosides flavonoids including dehydration and cross ring cleavage of the glucose moiety that producing 0, 2 cross ring cleavage [(M−H)-120] and 0, 3 cross ring cleavage [(M−H)-90] (Figueirinha et al., 2008). Compounds 7, 17 and 21 showed pseudomolecular ions at *m/z* 448, *m/z* 448 and *m/z* 431, respectively, and exhibited typical fragmentation patterns of *C*-glycosides, hence they were assigned as orientin, isoorientin and isovitexin, respectively.

Peaks 13 and 18 were di-*C*-glycosyl flavones and showed fragmentation pattern of [(M−H)-18], [(M−H)-90], [(M−H)-120], [aglycone + 113], and [aglycone + 83] found in negative mode MS/MS spectra (Zhang et al., 2011). Vicenin-2 (13) showed a

pseudomolecular ion at *m/z* 593 and characteristic fragment ions at 575 [(M−H)-18], 503[(M−H)-90], 473[(M−H)-120], and 383 [(M−H)-90–120].

Compound 18 was identified as isochaftoside (6-*C*-pentosyl-8-*C*-hexosyl apigenin) (Figueirinha et al., 2008; Zhang et al., 2011). It showed a pseudomolecular ion at *m/z* 564 and a fragmentation pattern typical of the asymmetric di-*C*-glycosides. MS2 data showed fragments at *m/z* 473[(M−H)-90] and 443 [(M−H)-120], indicating the presence of a *C*-hexosyl unit. In the same spectrum a fragment was observed at *m/z* 503 [(M−H)-60], corresponding to the fragmentation of a pentose. The base peak at *m/z* 473 [(M−H)-90] and the high abundance of the fragment at *m/z* 503 [(M−H)-60] revealed the presence of a 6-*C* pentosyl unit. The ions at *m/z* 353 (aglycone + 83) and 383 (aglycone + 113) supported the conclusion that apigenin (MW 270) was the aglycone.

Six compounds were identified as *O*-glycosyl flavones (peaks 9, 10, 28, 39, 40 and 41) and showed fragmentation pattern beginning with the cleavage of the *O*-sugar bond (Zhang et al., 2011). Five compounds were identified as *O*-, *C*-glycosyl flavones (compounds 11, 12, 14, 20 and 22) producing a characteristic fragment ions of *O*-, *C*-glycosyl flavones at [(M−H)-120], [(M−H)-90] and [(M−H)-162] or [(M−H)-146] (Figueirinha et al., 2008; Zhang et al., 2011). For example, isoorientin-2″-*O*-rhamnoside (20) had a pseudomolecular ion at *m/z* 593 that reveals a luteolin glycoside with a hexose and deoxyhexose. Fragments at *m/z* 447 [(M−H)-146], corresponding to loss of one deoxyhexose and another at *m/z* 429 [(M−H)-146-H_2O] corresponding to the loss of rhamnose + H_2O were observed. In addition, the absence of the aglycone ion is consistent with an *O*-, *C*-diglycoside structure (Figueirinha et al., 2008). MS^2 data also exhibited fragments at *m/z* 473 [(M−H)-120] (base peak) and a minor ion at *m/z* 503 [(M−H)-90], which indicated the presence of a *C*-glucosyl unit. Compound 11 (isovitexin-4′-*O*-glucoside) exhibited a pseudomolecular ion at *m/z* 593 with a fragmentation pattern of apigenin dihexoside and characteristic fragment ions of *O*-, *C*-glycosyl flavones with the loss of a *O*-hexosyl moiety (−162 μ).

Eighteen flavonols were identified in the ethanolic extract of the flowers of *S. securidaca*. The identification of these compounds was facilitated by the analysis of fragmentation pathways of $(M-H)^-/(M+H)^+$ ions in the negative and positive ion modes and the observation of glycosidic residues (rhamnosyl (146 μ) and glucosyl (162 μ)) were cleaved sequentially and generated characteristic aglycone fragments compared to the available literature. Among these compounds five compounds were identified as kaempferol glycosides (6, 15, 30, 32 and 35) and seven were identified as glycosides of quercetin (8, 24, 26, 31, 33, 38 and 45). In addition, four isorhamnetin glycosides (16, 19, 36 and 37) were identified.

Flavanones usually occur as *O*-glycosyl derivatives, with the sugar moiety bound to the aglycone hydroxyl group at either C-7 or C-3. Among these compounds, the *O*-diglycosides are a dominant category and their structures are usually characterized by the linkage of either neohesperidose or rutinose to the flavonoid skeleton. Compound 23 was found to be the neohesperidoside naringin. The precursor and product ions of this compound were *m/z* 579 and 271, respectively, indicating the loss of *O*-diglycoside (*m/z* 308) (Zhang et al., 2011) and compound 29 was found to be the rutinoside hesperidin with precursor and product ions of *m/z* 609 and 299, respectively.

One flavan-3-ol was identified, compound 5, which produced a protonated molecular ion peak at *m/z* (292) and yielded fragment ions at *m/z* 245, 205, and 179 characteristic for (+)-catechin. The fragment ion at *m/z* 245, corresponding to $[M+H-44]^+$, was produced by the loss of a $(CH)_2OH$ group from the catechin molecule (Sun et al., 2007).

Identification of phenolic acids

Eight phenolic acids belonged to various classes have been identified by comparing their retention times and fragmentation patterns with that reported (Sánchez-Rabaneda et al., 2003; Sun et al., 2007). In the positive ion mode hydroxybenzoic acids produced a protonated $[M+H]^+$ molecule and a $[M+H-44]^+$ fragment ion *via* loss of a CO_2 group from the carboxylic acid moiety (Sun et al., 2007). Three hydroxybenzoic acids have been identified; salicylic acid, gallic acid, and protocatechuic acid (2, 3, and 4).

Four hydroxycinnamic acids were identified; *trans*-cinnamic acid, caffeic acid, chlorogenic acid and *p*-coumaric acid (1, 44, 46 and 47). Caffeic and *p*-coumaric acids produced protonated molecular ions at *m/z* 179 and 165, respectively, and MS^2 spectra due to loss of CO_2 group from the carboxylic acid function (fragment ions at *m/z* 135 and 119, respectively, [(M−H)-44]) (Sun et al., 2007). Chlorogenic acid showed a molecular ion peak at (*m/z* 353) and a fragmentation ion that corresponding to the deprotonated quinic acid (*m/z* 191) (Sun et al., 2007). Compound 27 had an $[M+H]^+$ ion at *m/z* 303 which yielded a major ion at *m/z* 301 and minor ions at *m/z* 284, 257, and 229 characteristic of ellagic acid fragmentation (Sandhu and Gu, 2010).

Quantitative determination of some phenolic compounds in S. securidaca flowers

Absolute quantification of phenolics using the available standards was carried out (Table 2). Eight phenolic acids *i.e. trans*-cinnamic acid (2.36 ± 0.98 mg/100 g), salicylic acid (15.54 ± 1.91), protocatechuic acid (34.4 ± 0.155), ellagic acid (13.47 ± 3.95), caffeic acid (5.4 ± 1.43), chlorogenic acid (84.22 ± 2.08), *p*-coumaric acid (7.58 ± 1.51) and gallic acid (9.5 ± 0.14) were determined. In addition to seven flavonoids *i.e.* isoquercetrin (3340 ± 2.1), naringin (19.73 ± 3.016), hesperidin (32.098 ± 2.28), luteolin (10.247± 0.594), quercetin (1.16 ± 0.022), kaempferol (0.62 ± 0.129), catechin (39.44 ± 5.73) and hesperetin (0.109 ± 0.013).

Acute toxicity

The ethanolic extract of the flowers of *S. securidaca* was found to be safe up to a dose of 2 g/kg bwt with no mortality or signs of behavioral changes or toxicity observed which suggests its safety (Osweiler et al., 1985).

Table 2
Quantifications of some phenolic compounds identified in *S. securidaca* using HPLC analysis.

Compound	*Concentration (mg/100 g)
trans-cinnamic acid	2.36 ± 0.98
Salicylic acid	15.54 ± 1.91
Protocatechuic acid	34.4 ± 0.15
Naringin	19.73 ± 3.01
Ellagic acid	13.47 ± 3.95
Luteolin	10.24 ± 0. 59
Isoquercetrin	3340 ± 2.1
Quercetin	1.16 ± 0.02
Kaempferol	0.62 ± 0.12
Caffeic acid	5.4 ± 1.43
Catechin	39.44 ± 5.73
Hesperidin	32.09 ± 2.28
p-Coumaric acid	7.58 ± 1.51
Hesperetin	0.10 ± 0.01
Gallic acid	0.95 ± 0.014
Chlorogenic acid	8.42 ± 2.08

*Average concentration of three HPLC determinations ± SD.

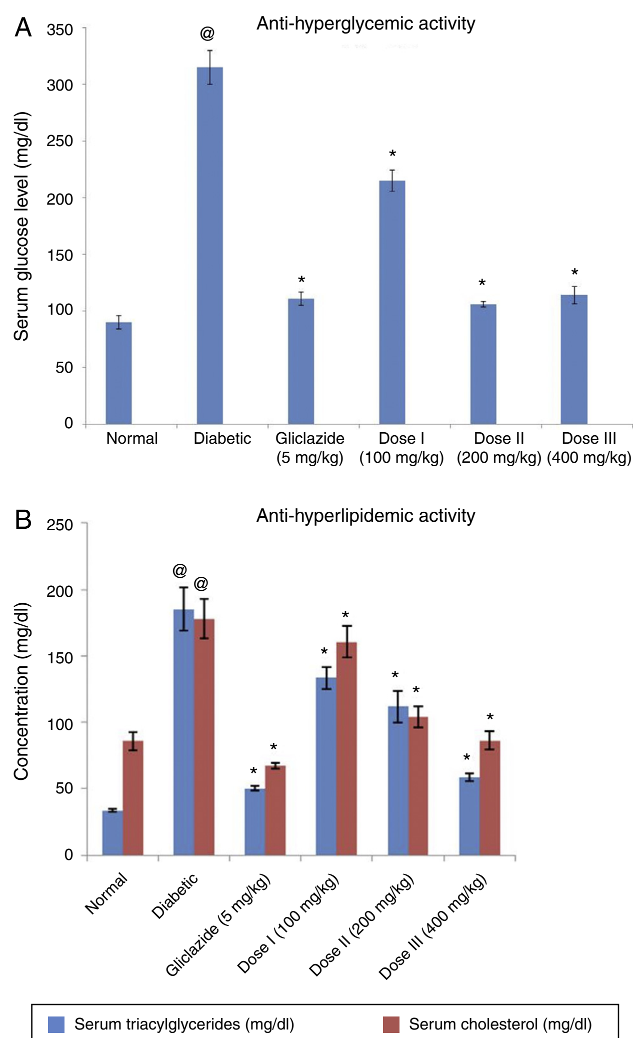

Fig. 2. Biological activities of the ethanolic extract of the flowers of *S. securidaca* L. (A) Effect on serum glucose level in alloxan-induced hyperglycemic rats. (B) Serum triacylglycerides and cholesterol levels in alloxan-induced hyperglycemic rats. @Significant difference from normal rats $p < 0.05$, *Significant difference from hyperglycemic rats $p < 0.05$.

Anti-hyperglycemic activity

The ethanolic extract of the flowers showed marked anti-diabetic activity on blood glucose levels in alloxan-induced diabetic rats at all tested doses 100, 200 and 400 mg/kg bwt with potencies of 31.78, 66.41 and 63.8% respectively (Fig. 2A). The most potent reduction in serum glucose level was recorded with a dose of 200 mg/kg bwt compared to Gliclazide at a dose of 5 mg/kg bwt (64.85%).

Anti-hyperlipidemic activity

The ethanolic extract of the flowers exhibited potent hypolipidemic effect on the elevated serum triacylglycerides and cholesterol levels in alloxan induced hyperglycemic rats (Fig. 2B) with 39.68% and 41.46% decreases in serum triacylglycerides and cholesterol levels at dose 200 mg/kg, 68.46% and 51.50% for dose 400 mg/kg, compared to gliclazide at dose of 5 mg/kg bwt showing reduction in serum triacylglycerides and cholesterol levels with 73.01% and 62.20%, respectively.

Diabetes mellitus is a complex disorder that characterized by chronic hyperglycemia and dyslipidemia. The disease becomes a real problem of public health in developing countries, where its prevalence is increasing steadily and adequate treatment is often expensive or unavailable. Results of the present study revealed that alloxan-induced hyperglycaemia is associated with metabolic changes. Alloxan induces chemical diabetes in rats by damaging insulin secreting pancreatic β-cells leading to decrease in insulin release (Kim et al., 2006).

Hyperlipidaemia is a major characteristic of diabetes (Pushparaj et al., 2000). DM induced hyperlipidaemia is attributable to excess mobilization of fat from the adipose tissue due to the utilization of the glucose. Moreover, studies suggested that hyperlipidemia is one of the most common features in alloxan-induced hyperglycaemia in experimental rats (Krishnakumar et al., 2000). In this study, an increase in the levels of total cholesterol and triglycerides has been observed in alloxan-induced hyperglycaemic rats. Plants used in traditional medicine to treat diabetes mellitus represent a valuable alternative for the control of this disease (Kumar and Verma, 2011). In the present study, we evaluate the acute toxicity, anti-hyperglycemic and hypolipidemic effects of the ethanolic extract of the flowers of *S. securidaca* L. Moreover, the phenolic composition of the extract was characterized using HPLC-DAD-ESI/MS technique to help in chemical profiling and standardization of the extract. Oral treatment of hyperglycemic rats with the ethanolic extract of the flowers (100, 200 and 400 mg/kg bwt) significantly decreased the elevated serum glucose level as well as serum total cholesterol and triglycerides levels in diabetic rats with potencies comparable to gliclazide. Hence, we could say that extract had beneficial effects on carbohydrate metabolism in hyperglycaemic rats.

HPLC-DAD-ESI/MS analysis revealed that the ethanolic extract contains complex mixture of phenolic compounds including different classes of phenolic acids and flavonoids. Most of the detected phenolic compounds were reported to have anti-diabetic effect though different mechanisms. Isovitexin, luteolin 7-*O*-glucoside, hyperoside and isorientin were reported to possess antihyperglycemic action (Brahmachari, 2011; del Pilar Nicasio-Torres et al., 2012; Folador et al., 2010). Vicenin-2 was reported to be an antioxidant that strongly inhibited α-glucosidase and exhibited potent anti-glycation properties (Islam et al., 2014). Isoquercetrin and astragalin were found to be glycation inhibitors having comparable activity to that of aminoguanidine (Brahmachari, 2011). Rutin was reported to possess potent hypoglycemic and hypolipidemic activities by enhancing peripheral glucose utilization by skeletal muscle and stimulation of β-cells (Jadhav and Puchchakayala, 2012).

The respective aglycones, quercetin and kaempferol were found to improve insulin-stimulated glucose uptake in mature adipocytes (Figueirinha et al., 2008). Isorhamnetin-3-*O*-glucoside was reported to lower serum glucose concentration, sorbitol accumulation in the lenses, red blood cells by exerting potent inhibitory activity against rat lens aldose reductase, leading to improved diabetic complications (Brahmachari, 2011). Rutin and hesperidin were reported to prevent the progression of hyperglycemia by increasing hepatic glycolysis, glycogen concentration and lowering hepatic gluconeogenesis (Jung et al., 2004). Through a docking study, catechin showed potential agonist characteristic to insulin receptor (insulin mimetic) (Pitchai and Manikkam, 2012). Gallic acid was reported to have hypoglycemic and hypolipidaemic effects against streptozotocin induced diabetic rats (Latha and Daisy, 2011), while chlorogenic acid was reported to exhibit hypoglycemic, hypolipidemic, and antioxidant properties (del Pilar Nicasio-Torres et al., 2012).

Conclusion

In this work we have evaluated the anti-hyperglycemic and anti-hyperlipidemic activity of the ethanolic extract of *S. securidaca*

flower. Phenolic acids and flavonoids extracted with 90% ethanol have been identified and quantified. The ethanolic extract of the flowers was safe up to dose of 2 g/kg. The extract showed potent anti-diabetic and hypolipidemic effect in alloxan induced hyperglycemic in rats. The current results indicate that the flavonoid- and phenolic acid-rich extract of *S. securidaca* flowers is a promising natural pharmaceutical for combating diabetes.

Conflicts of interest

The authors declare that they have no competing interests.

Author's contributions

RI wrote the manuscript, carried out extraction procedures and analyzed data. AM wrote the manuscript, planed the work and analyzed data. DS carried out biological activity. EN carried out LCMS analysis and interpreted data. AE revised the manuscript and supervised work. SE suggested the point and revised the manuscript.

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